Spatiotemporal distribution of small ubiquitin-like modifiers during human placental development and in response to oxidative and inflammatory stress

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Key points

- The post-translational modification of target proteins by SUMOylation occurs in response to stressful stimuli in a variety of organ systems.
- Small ubiquitin-like modifier (SUMO) isoforms 1–4 have recently been identified in the human placenta, and are upregulated in the major obstetrical complication of pre-eclampsia.
- This is the first study to characterize the spatiotemporal distribution of SUMO isoforms and their targets during placental development across gestation and in response to stress induced by pre-eclampsia and chorioamnionitis.
- Keratins were identified as major targets of placental SUMOylation. The interaction with SUMOs and cytoskeletal filaments provides evidence for SUMOylation possibly contributing to underlying dysfunctional trophoblast turnover, which is a hallmark feature of pre-eclampsia.
- Further understanding the role of individual SUMO isoforms and SUMOylation underlying placental dysfunction may provide a target for a novel therapeutic candidate as an approach for treating pre-eclampsia complicated with placental pathology.

Abstract SUMOylation is a dynamic, reversible post-translational modification that regulates cellular protein stability and localization. SUMOylation occurs in response to various stressors, including hypoxia and inflammation, features common in the obstetrical condition of pre-eclampsia. SUMO isoforms 1–4 have recently been identified in the human placenta, but less is known about their role in response to pre-eclamptic stress. We hypothesized that SUMOylation components have a unique spatiotemporal distribution during placental development and that their subcellular localization can be further modulated by extra-cellular stressors. Placental SUMO expression was examined across gestation. First-trimester human placental explants

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and JAR cells were subjected to hypoxia or TNF-α cytokine, and subcellular translocation of SUMOs was monitored. SUMOylation target proteins were elucidated using mass spectrometry and proximity ligation assay. Placental SUMO-1 and SUMO-4 were restricted to villous cyto-trophoblast cells in first trimester and syncytium by term, while SUMO-2/3 staining was evenly distributed throughout the trophoblast across gestation. In placental villous explants, oxidative stress induced hyperSUMOylation of SUMO-1 and SUMO-4 in the syncytial cytoplasm, whereas SUMO-2/3 nuclear expression increased. Oxidative stress also upregulated cytoplasmic SUMO-1 and SUMO-4 protein expression ($P < 0.05$), similar to pre-eclamptic placentas. Keratins were identified as major targets of placental SUMOylation. Oxidative stress increased the cytokeratin-7 to SUMO-1 and SUMO-4 interactions, while inflammatory stress increased its interaction with SUMO-2/3. Overall, SUMOs display a unique spatiotemporal distribution in normal human placental development. Our data indicate SUMOylation in pre-eclampsia, which may impair the stability of cytoskeleton filaments and thus promote trophoblast shedding into the maternal circulation in this condition.

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Introduction

The small ubiquitin-related modifier (SUMO) peptide is reversibly conjugated to proteins resulting in a post-translational protein modification that is implicated in a number of different cellular processes including DNA repair, cell cycle progression and the regulation of a protein's function, localization and stability. Although both SUMO and ubiquitin act as post-translational modifiers, sometimes even competing for the same target, SUMOylated proteins are not usually targeted for degradation to the same extent ubiquitinated proteins are (Gill, 2004). SUMO homeostasis is critical for cell function, as its disruption has been linked to cancer development and progression (Bawa-Khalfe & Yeh, 2010), deregulation of mitosis (Dasso, 2008) and neurodegenerative disease (Anderson *et al.* 2009). Mammals express at least four SUMO isoforms (Hay, 2005) and emerging evidence has potentially identified a novel fifth SUMO isoform (Liang *et al.* 2016). SUMO peptides are small proteins (76–100 aa) and 10–12 kDa in size (Geiss-Friedlander & Melchior, 2007). SUMO-2 and SUMO-3 share 97% homology and as such are most often referred to as SUMO-2/3 (Scioscia *et al.* 2011). SUMO-1, SUMO-2 and SUMO-3 are ubiquitously expressed in all eukaryotic cells, whereas SUMO-4 expression is restricted to the kidney (Chen *et al.* 2014), immune cells, pancreas (Wang & She, 2008) and, discovered most recently, the placenta (Baczyk *et al.* 2017). In most cases, SUMOs are covalently conjugated to their targets in a series of conserved steps, involving activating and conjugating enzymes and ligase proteins. UBC9 is classified as the main conjugation enzyme (Hay, 2005). DeSUMOylation, or the cleavage of the covalent conjugation between a C-terminal glycine residue of SUMO peptides and ε-amino group of lysine residue of the target protein, is achieved by a family of sentrin/SUMO-specific proteases (SENPs) (Yeh *et al.* 2000). Since SUMO peptides can multimerize and form polymeric chains (Tatham *et al.* 2001; Matic *et al.* 2008), in addition to monoSUMOylation, substrates can be modified by multiSUMOylation or polySUMOylation (Pichler *et al.* 2017).

The placenta is a highly specialized organ responsible for the delivery of oxygen and nutrients and for the removal of waste products from the developing fetus. The outer epithelial layer of the placenta, which is in contact with maternal blood, is known as the villous trophoblast and carries out these functions. This trophoblast layer is composed of proliferating cytotrophoblast cells, which give rise to the overlying terminally differentiated syncytiotrophoblast cell layer. Aberrant trophoblast turnover has been linked to placental dysfunction underlying various obstetrical conditions (Walker *et al.* 2012). Disruption of SENP1 and SENP2 activity in mice results in impaired placental trophoblast development and function affecting embryonic viability due to hyperSUMOylation (Yamaguchi *et al.* 2005; Chiu *et al.* 2008). SUMOylation has been implicated in the alteration of placental transcription factors regulating trophoblast differentiation including glial cell missing-1 (GCM1) (Chou *et al.* 2007), downstream regulatory element antagonist modulator (DREAM; also referred to as KChIP-3 or calsenilin) (Palczewska *et al.* 2011), p53 (Chiu *et al.* 2008) and hypoxia inducible factor-1α (HIF-1α) (Bhattacharjee *et al.* 2016) by affecting protein stability, activity and intracellular localization. These transcription factors are of particular interest as they have also been shown to be involved in pre-eclampsia disease progression (Chen *et al.* 2004; Baczyk *et al.* 2013*b*; Sharp *et al.* 2014). Furthermore, we have recently demonstrated

that pre-eclamptic placentas exhibit hyperSUMOylation (Baczyk *et al.* 2013*a*, 2017), further suggesting that the dynamic regulation of SUMOs is critically linked to placental function.

Initially SUMOylation was thought to primarily affect transcription factors alone. However, new evidence suggests that SUMOylation is also involved in alternative splicing (Sánchez-Alvarez *et al.* 2010) and plays important roles outside of the nucleus (Wasik & Filipek, 2014), including the regulation of nucleocytoplasmic shuttling of proteins (Wang *et al.* 2012; Zhang *et al.* 2014), receptor function (Wasik & Filipek, 2014), mitochondrial dynamics, autophagy (Mayo, 2012) and cytoskeletal function (Alonso *et al.* 2015). Additionally, spatiotemporal cellular distribution of SUMO components has been implicated in the development of the mouse brain (Hasegawa *et al.* 2014), rat central nervous system (Loriol *et al.* 2012) and *Drosophila* germ line (Hashiyama *et al.* 2009). To date, the cellular distribution of SUMO iso-forms and their underlying role in placental development remain unknown.

Here we investigated the subcellular localization of SUMO isoforms in healthy and pathological placentas by monitoring their intra- and extracellular distribution throughout pregnancy. In pre-eclampsia, placentas are exposed to increased level of oxidative stress, due to ischaemia–reperfusion injury that occurs with uteroplacental vascular insufficiency (Burton & Jauniaux, 2011). As such, we used hypoxia and H_2O_2 to determine the effects of oxidative stress on SUMOylation. The effects of tumour necrosis factor-α (TNF-α) were also examined as a marker of inflammatory stress, as TNF-α is elevated in conditions such as severe chorioamnionitis (Zhang *et al.* 2000). Thus, we hypothesized that a distinctive spatiotemporal distribution of SUMO isoforms occurs across gestation and in response to stressful cellular stimuli.

Methods

Tissue collection and ethical approval

Placental villous samples from the first and second trimester of pregnancy were obtained from Morgentaler Clinic (Toronto, Canada), following a voluntary legal termination of pregnancy. Second-trimester, third-trimester and pathology samples were collected from deliveries at Mount Sinai Hospital (Toronto, Canada) between 2004 and 2009. Research Ethics Board approval (11-0248-E) was obtained for this study and all patients gave written informed consent. These methods utilized comply with *The Journal of Physiology*'s policies and conforms to the standards set by the *Declaration of Helsinki*. Gestational age and viability were established pre-operatively by ultrasound. The patient population

used in this study overlaps with the patient pool used in our previous studies (Drewlo *et al.* 2011; Czikk *et al.* 2013; Baczyk *et al.* 2017).

Immunofluorescence

Immunofluorescence was performed either on the Research Centre for Women's and Infants' Health (RCWIH) BioBank tissue array (TMA no. 2 array; http://biobank.lunenfeld.ca/?page=Tissue%20arrays), which contained placental samples from 4 to 37 weeks of gestation, or on first-trimester villous placental explants. First-trimester villous placental explants were cultured under control conditions (normoxia, 8% O_2) and were compared to oxidative stress (by severe hypoxia of 0.5% O_2; 24 h or 0.1 mM H_2O_2; 1 h) or inflammatory stress (TNF-α 10 ng ml^{-1}; 24 h). Paraformaldehyde-fixed (4%) placental tissue was dehydrated and paraffin-embedded. Immunofluorescence was performed on rehydrated sections. Following blocking with Dako blocking solution (Dako, Carpinteria, CA, USA), primary antibodies to SUMO-1 (1:100 dilution; Abcam, Cambridge, MA, USA), SUMO-2/3 (1:100 dilution; Abcam) and SUMO-4 (1:500 dilution; Abcam) were applied to sections overnight at 4°C. The following day the signal was amplified with anti-rabbit 488 and/or anti-mouse 546 antibodies (1:300 dilution; Invitrogen, Burlington, ON, Canada) and tissues were counter stained with 4$'$,6-diamidino-2-phenylindole (DAPI; 1:1000 dilution, Sigma-Aldrich, Oakville, ON, Canada). Negative controls included omission of the primary antibody and use of non-specific matched IgG. Slides were visualized using spinning disc confocal micro-scopy (DMI6000B; Leica Microsystems, Concord, ON, Canada). Images were taken under the same acquisition settings. Following immunostaining of tissue arrays, two independent reviewers blindly assessed the proportion of SUMO-positive cytotrophoblast and syncytiotrophoblast nuclei and the total proportions were averaged.

Cellular fractionation

The trophoblast JAR choriocarcinoma cell line was used to examine oxidative stress by treatment with 1% O_2 or 0.1 mM H_2O_2 and inflammatory stress by treatment with 10 ng ml^{-1} TNF-α for 4 h. JAR cells were grown and treated in 10 mm dishes. Nuclear and cytoplasmic extracts were prepared using Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, cells were washed in ice cold PBS and cytoplasmic fractions were collected following incubation with supplied Hypotonic Buffer. Following a 30 s spin, remaining nuclei were lysed and proteins solubilized in the provided lysis buffer. The protein concentration of the cell extracts was measured using Pierce BCA Protein Assay (Thermo Fisher Scientific, Ottawa, ON, Canada).

The efficiency of cellular fractionation was assessed using nucleus-specific and cytoplasm-specific antibodies, lamin B and α-tubulin, respectively.

Western blot

Twenty-five micrograms of protein and $4\times$ loading dye with 10% β-mercaptoethanol were electrophoresed in 4–20% Mini-PROTEIN TGX^{TM} pre-cast gels (Bio-Rad, Mississauga, ON, Canada). Following electrophoresis, proteins were transferred onto 0.2 μm polyvinylidene fluoride membrane, Trans-Blot Turbo^{TM} transfer pack (Bio-Rad), which was used according to the manufacturer's specifications. Following blocking in 5% milk/Tris-buffered saline and Tween 20 (TBST), membranes were incubated with primary antibodies overnight at 4°C (SUMO-1, 1:1000 dilution; SUMO-2/3, 1:500 dilution; SUMO-4, 1:2000 dilution; lamin B, 1:500 dilution; α-tubulin, 1:5000 dilution). Following washing, membranes were incubated in respective secondary horseradish peroxidase-conjugated antibodies (GE Healthcare UK Ltd, Little Chalfont, UK) and developed using Western Lightning Plus-ECL (Thermo Fisher Scientific) on autoradiography film (Denville Scientific, South Plainfield, NJ, USA). Band intensities, within the linear range, were quantified using Quantity One Software (Bio-Rad). Levels of protein of interest were normalized to housekeeping proteins and further compared to its respective controls set as 1.

Affinity purification

In brief, full-length human SUMO-1, SUMO-2 and SUMO-4 were cloned in our pcDNA5 FRT/TO FLAGBirA* expression vector, to allow biotinylation of the bait and proximal interactors (Roux *et al.* 2012). Samples were generated by transient transfection of the FlagBirA*-tagged SUMO moieties in a 60% confluent 150 mm diameter culture dish of JAR cells, incubated for 24 h to allow proper expression, then for an additional 24 h with biotin- (50 μM, BioShop, Burlington, ON, Canada) and tetracycline- (1 μg ml^{-1}, Sigma-Aldrich) containing media. Osmotic stress was induced by adding 500 mM NaCl supplemented complete media for 15 min. Cells were collected and pelleted (600 g, 3 min), the pellet was washed twice with PBS, and dried pellets were snap frozen. Pellets were lysed in 5 ml of RIPA lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.1% SDS, 1:500 protease inhibitor cocktail (Sigma-Aldrich), 250 U Turbonuclease (Accelagen, San Diego, CA, USA)) at 4°C for 1 h, then sonicated (30 s, at 35% power, Sonic Dismembrator 500; Fisher Scientific, Ottawa, ON, Canada) to completely disrupt visible aggregates. The lysate was centrifuged at 35,000 g for 30 min. Clarified supernatants were incubated with 30 μl packed, pre-equilibrated streptavidin–sepharose beads (GE Healthcare) at 4°C for 3 h. Beads were collected by centrifugation (600 g, 2 min), washed 6 times with 50 mM ammonium bicarbonate pH 8.2, and treated with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-trypsin (Promega, Fitchburg, WI, USA, 16 h at 37°C). The supernatant containing the tryptic peptides was then collected and lyophilized. Peptides were resuspended in 0.1% formic acid and one-seventh of the sample (representing less than one plate of cells) was analysed per mass spectrometry run.

Mass spectrometry

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was conducted using a 120 min reversed-phase buffer gradient running at 250 nl min^{-1} on a Proxeon EASY-nLC pump in-line with a hybrid LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). Biotinylated proteins were identified by tandem mass spectrometry (Coyaud *et al.* 2015). Regular BioID background was removed using HEK293 cells expressing BirA* tag alone, and cell type-specific background using FlagBirA*–green fluorescent protein (GFP) as a bait protein. For protein identification, raw files were converted to the mzXML format using Proteowizard open source software (Kessner *et al.* 2008), then searched using X!Tandem (Craig & Beavis, 2004) against Human RefSeq Version 45. Data were analysed using the trans-proteomic pipeline (TPP) (Pedrioli, 2010) via the ProHits 2.0.0 software suite (Liu *et al.* 2010). Cytoskeleton IDs of the keratin, tubulin and actin families were added up for comparison (data not shown).

Proximity ligation assay

To validate the findings that SUMO placental isoforms interact with cytokeratin-7 (Ct-7), placental JAR cells were subjected to a proximity ligation assay (Ristic *et al.* 2016). JAR cells were grown overnight on chamber slides (Lab Tek, Thermo Fisher Scientific) to 50% confluency. Cells were then treated for 4 h with 0.1 mM H_2O_2 or 10 g ml^{-1} of TNF-α. Following treatments, JARs were fixed with ice cold methanol–acetone (1:1) for 5 min, followed by 0.02% Triton X-100 permeabilization for 3 min after blocking with the provided blocking solution from the Duolink *in situ* assay (Sigma-Aldrich). Dual immunocytochemistry with individual SUMO components and Ct-7 was set up with antibodies to SUMO-1 (1:100 dilution; Abcam) and SUMO-2/3 (1:100 dilution; Abcam), SUMO-4 (1:500 dilution; Abcam) and Ct-7 (1:100 dilution; Dako) for 2 h at 37°C. The slides were imaged using the WaveFX Spinning Disc Confocal System by Quorum (Guelph, ON, Canada) with optimized Yokogawa CSU X1, Hamamatsu Photonics (Hamamatsu City, Japan) EM-CCD digital camera

Image EM (C9100-13), and a Leica (Wetzlar, Germany) DMI600B inverted research grade motorized microscope run by Velocity 6.3.0 Acquisition software.

Statistical analysis

All experiments were performed in technical triplicates of at least biological quadruplicates. Two-way ANOVA with Bonferroni's multiple comparison test was used to compare the proportion of SUMO-1 to SUMO-4 positive cytotrophoblast and syncytiotrophoblast nuclei over the course of human gestation. A one-sample Student's t test was used to compare SUMO nuclear and cytoplasmic protein expression levels compared to their corresponding vehicle/control set as 1. All statistical calculations were performed using Prism 4 software (GraphPad Software, La Jolla, CA, USA). Data are presented as means $\pm$ standard error of the mean. Significance was considered at P values ≤ 0.05.

Results

Spatiotemporal distribution of SUMOs during human placental development

To evaluate SUMOs in placental development, we characterized the spatiotemporal distribution of the SUMO isoforms in the trophoblast layer, utilizing placental sections from each trimester across gestation. During the first and second trimester, cytotrophoblast cells were mainly positive for SUMO-1 and SUMO-4, whereas by term the syncytiotrophoblast layer was equally positive (Fig. 1A). Conversely, SUMO-2/3 immunostaining was evenly distributed between cytotrophoblast cells and the syncytiotrophoblast layer throughout gestation (Fig. 1A). SUMO-1 had significantly higher cytotrophoblast compared to syncytiotrophoblast expression until 30–34 weeks' gestation ($P < 0.05$; Fig. 1B). SUMO-2/3 appears to be constitutively expressed in cytotrophoblast cells throughout gestation (Fig. 1C). Similar to SUMO-1, SUMO-4 was also highly expressed in cytotrophoblasts earlier in gestation with equal distribution by term ($P < 0.05$; Fig. 1D). These observations suggest that SUMO isoforms each have a unique subcellular distribution within the trophoblast underlying healthy placental development and function.

Oxidative and inflammatory stress induces specific compartmentalization of SUMOs within the trophoblast layer

To investigate the potential mechanisms accounting for the differences in the spatiotemporal distribution of SUMO isoforms in the trophoblast layer over gestation, we tested the hypothesis that stressful stimuli differentially regulate SUMO isoform placental expression. Exposure of placental villous explants to severe hypoxia caused a strong shift of SUMO-1 and SUMO-4 immunostaining from the cytotrophoblast nucleus to the cytoplasm, as well as into the syncytiotrophoblast layer (Fig. 2). In response to hypoxia, SUMO-2/3 immunostaining showed increased cytotrophoblast nuclear expression (Fig. 2). Exposure of first trimester placental explants to 0.1 mM H_2O_2 resulted in analogous SUMO-1 and SUMO-4 cytoplasmic distribution, with increased SUMO-1 trophoblast perinuclear expression (Fig. 2). Cytokine treatment with TNF-α resulted in equal accumulation of SUMO-1 and SUMO-2/3 across the trophoblast layer, whereas SUMO-4 showed predominantly cytotrophoblast accumulation. However, SUMO-2/3 had the most pronounced pattern with TNF-α treatment, where all of the immunostaining was confined to the cytoplasm (Fig. 2). Since cytoplasmic staining of SUMO-1 and SUMO-4 is observed under oxidative stress and cytoplasmic SUMO-2/3 is observed only under cytokine treatment, this further supports our hypothesis that individual SUMO isoforms have distinctive expression in both healthy placenta and in response to stressful stimuli.

Oxidative (hypoxia and H₂O₂) and inflammatory (TNF-α) stress influences SUMO protein expression

To accurately assess the nuclear *versus* cytoplasmic SUMO protein levels following stress, we performed cell fractionation prior to western blotting (Fig. 3A). As predicted from our observations in first-trimester explants (Fig. 2), both oxidative stress and cytokine treatment of cells led to differential profiles of subcellular SUMO isoform localization. TNF-α treatment of JAR cells demonstrated a trend towards elevated levels of cytoplasmic and nuclear SUMO-2/3 compared to control (Fig. 3B). Hypoxic treatment of JAR cells did not result in elevated cytoplasmic SUMO-2/3, but rather caused a significant increase in nuclear SUMO-2/3 (3.0 ± 0.8-fold, $P < 0.01$, $n = 4$; Fig. 3C). As compared to controls, hypoxic treatment resulted in elevated cytoplasmic levels of SUMO-4 (2.3 ± 0.3-fold, $P < 0.05$, $n = 4$; Fig. 3C). Peroxide treatment of JAR cells resulted in elevated cytoplasmic levels of SUMO-4 (2.7 ± 0.3-fold, $P < 0.05$, $n = 4$) and reduced nuclear levels of SUMO-1 (0.4 ± 0.1-fold, $P < 0.05$; $n = 4$) and SUMO-2/3 (0.3 ± 0.1-fold, $P < 0.01$; $n = 4$) as compared to controls (Fig. 3D). Consistently, oxidative stress was more robust at inducing cytoplasmic SUMO-4 expression.

Identification of placenta-specific targets of SUMOylation

To identify stress-induced SUMO conjugates in placental cells, we used an *in vivo* proximity-dependent
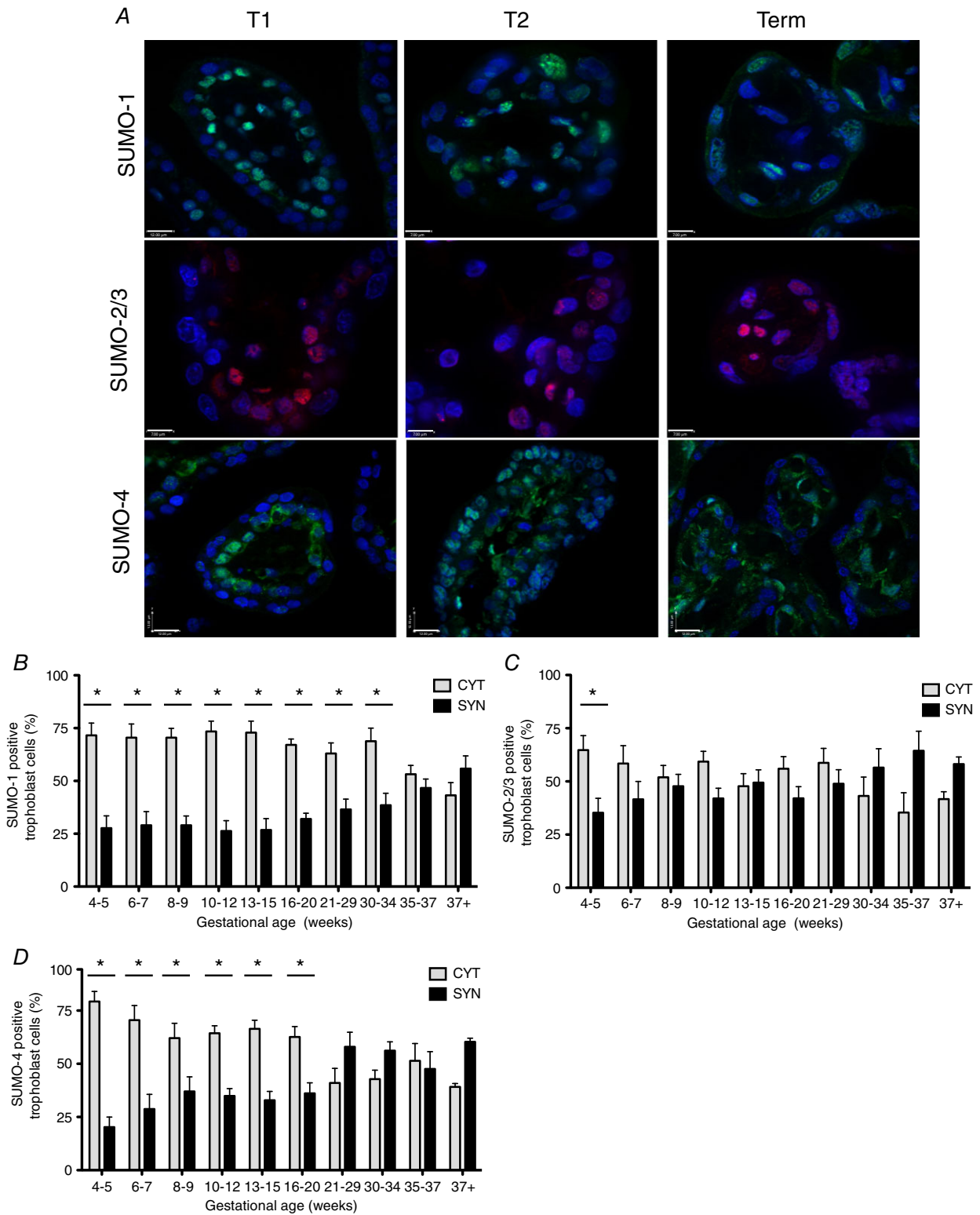
Figure 1. Spatiotemporal distribution of SUMOs across gestation
A, representative images of SUMO-1 (green), SUMO-2/3 (red) and SUMO-4 (green) immunofluorescence from 7-, 18- and 37-week placentas (nuclear counter-stained with DAPI). Scale bars: 12 μm. *B–D*, the proportion of SUMO-1 (*B*), SUMO-2/3 (*C*) and SUMO-4 (*D*) positive cytotrophoblast (CYT) *vs.* syncytiotrophoblast (SYN) nuclei. During first and second trimester, CYT cells are mainly positive for SUMO-1 and SUMO-4, whereas by term the

biotinylation (BioID) in placental JAR cells. One hundred and seven high confidence proteins were identified, 24 in SUMO-1, 19 in SUMO-2/3 and 85 in SUMO-4 (with a false discovery rate of 1%). We detected SUMO system components (SAE1, UBE2I) and several reported SUMO substrates (TOP2B, TRIM33, SRSF1, PPIG, ZNF687, KRT17; Hendriks *et al.* 2015). Strikingly, the cytokeratin genes were the most represented family (19/107), including trophoblast-specific keratin such as KRT7 (Ct-7).

Encompassing all detected peptides in the raw data, we estimated the relative abundance of three main families of cytoskeleton filaments, namely keratin as well as tubulin and actin (Table 1). While no changes were observed in the relative abundances of actin or tubulins, cytokeratins (peptide counts of 42 keratin gene family members aggregated) were highly represented under standard culture conditions, and their relative abundance doubled in SUMO-1 (2000 to >5000 peptides) and SUMO-4 (2500 to >7000 peptides) BioID data upon stress (proportionally representing an increase from 20% to 40% of all detected peptides between controls *vs.* stressed samples) (Fig. 4 and Table 1). No modification was observed in GFP and SUMO-2 interactomes (Fig. 4 and Table 1).

SUMO isoform-specific interaction with cytokeratin-7 upon stress

Our mass spectrometry data revealed keratins as a major candidate for SUMOylation in placenta. As such, placental
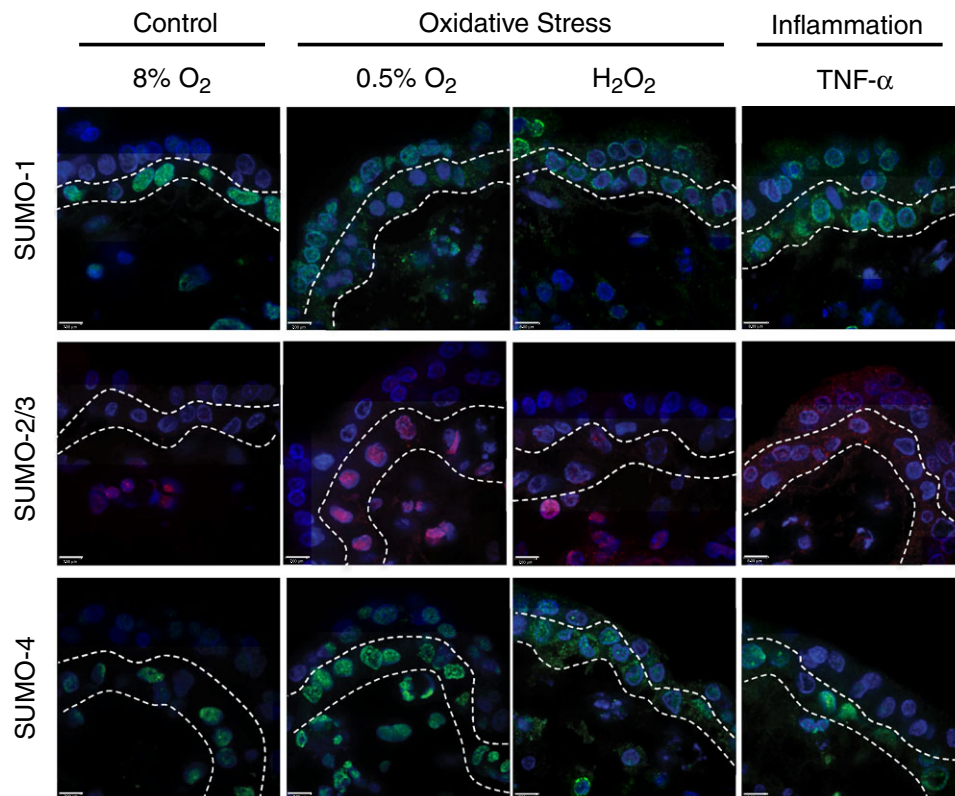
Figure 2. Oxidative and inflammatory stress induced isoform-specific SUMO distribution within the trophoblast layer of first trimester placental explants

Placental explants were cultured under control (normoxic 8% O_2), oxidative stress (by severe hypoxia 0.5% O_2 and 0.1 mM H_2O_2) and inflammatory stress (10 ng ml^{-1} TNF-α) for 24 h. Exposure of oxidative and inflammatory stress resulted in perinuclear and cytoplasmic SUMO-1 localization. SUMO-2/3 staining in the syncytium in response to hypoxia elevated in cytotrophoblast nuclei. TNF-α induced strong SUMO-2/3 cytoplasmic staining throughout the trophoblast layer including syncytium. SUMO-4 immunostaining was demonstrated in cytoplasm, especially within the cytotrophoblast layer when treated with oxidative stress. Inflammatory stress from TNF-α had negligible effects. White dashed lines outline the cytotrophoblast layer from the syncytium (above) and stroma (below). Scale bar: 8 μm.

JAR cells were subjected to a proximity ligation assay to validate the interaction between SUMOs and Ct-7. Under control conditions, we observed weak SUMO and Ct-7 co-localization. However, H_2O_2 treatment resulted in a dramatic increase in SUMO-1 and SUMO-4 co-localization with Ct-7 (Fig. 5). Conversely, TNF-α treatment resulted in an increased SUMO-2/3 and Ct-7 interaction (Fig. 5). Specificity of each SUMO and Ct-7 interaction was validated using single antibody controls in the proximity ligation assay, which was devoid of any immunofluorescence (data not shown). Therefore, our

results suggest that oxidative stress induces SUMOylation of Ct-7 by SUMO-1 and SUMO-4, but not SUMO-2.

Placentas from pregnancies complicated by severe pre-eclampsia or chorioamnionitis display a unique SUMO distribution

Since oxidative and inflammatory stress of placental explants stimulated distinct SUMO subcellular localization, we examined the SUMO subcellular distribution within the trophoblast layer in related placental
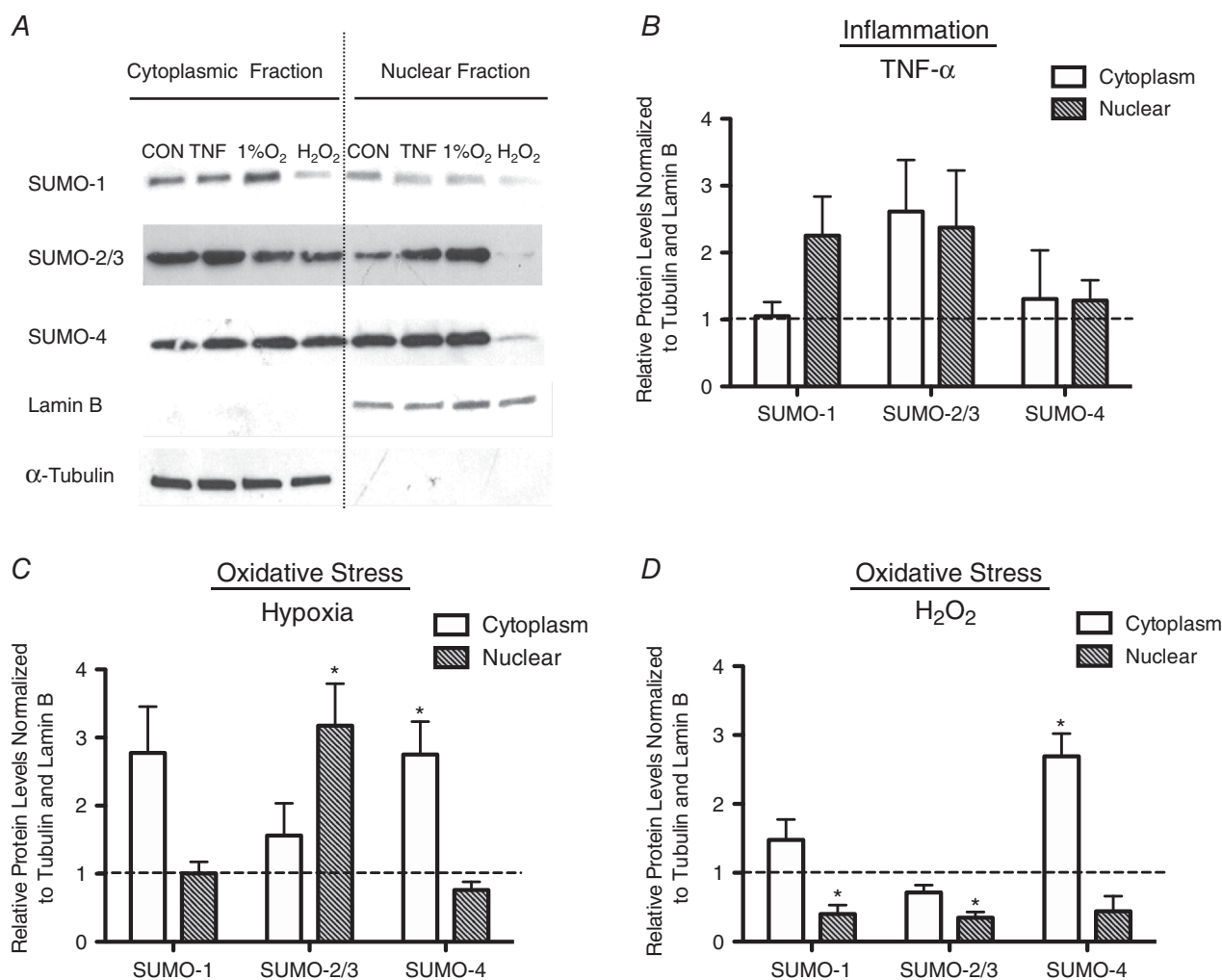

Figure 3. Subcellular localization of SUMO components in response to inflammatory and oxidative stress in trophoblast JAR cells
Representative western blot images (*A*) and relative protein levels (*B–D*) for subcellular translocation of SUMOs treated with 10 ng ml^{-1} TNF-α (inflammatory stress) (*B*), hypoxia (1% O_2; oxidative stress) (*C*) or 0.1 mM H_2O_2 (oxidative stress) (*D*) for 4 h. Protein levels were normalized to either lamin B or α-tubulin and treatment effects were expressed relative to control (untreated cells) set as 1 (dashed line). SUMO-1 demonstrated a trend to increased cytoplasmic expression with hypoxic stimuli. SUMO-2/3 protein levels varied in response to oxidative stress, but was trending up in both nuclear and cytoplasmic expression in response to inflammatory stress. SUMO-4 demonstrated increased cytoplasmic expression in response to oxidative stress ($P < 0.05$). Values presented as means $\pm$ SEM, $n = 4$; significance shown as *$P < 0.05$. C, control; TNF, TNF-α; 1%, 1% O_2.

pathologies. Additionally, we stained these pathological placentas for the identified SUMO modified target, trophoblast-specific Ct-7. We observed strong cytoplasmic and perinuclear staining of SUMO-1 and SUMO-4 in pre-eclamptic placentas, whereas SUMO-2/3 expression was predominantly restricted to nuclei (Fig. 6). This

trend was reversed in chorioamnionitis, where placentas demonstrated nuclear SUMO-1 and SUMO-4 expression and cytoplasmic SUMO-2/3 staining (Fig. 6). Immunostaining in placentas from age-matched control and severe chorioamnionitis patients displayed distinctive and strong Ct-7 in the trophoblast layer. In contrast, pre-eclamptic placentas showed reduced and irregular expression of Ct-7 within the trophoblast layer. Additionally, pre-eclamptic placentas displayed co-localization of SUMO-4 and Ct-7 (Fig. 6). Thus, these two discrete placental pathologies show very distinct patterns of expression and interactions between the SUMOs and Ct-7.
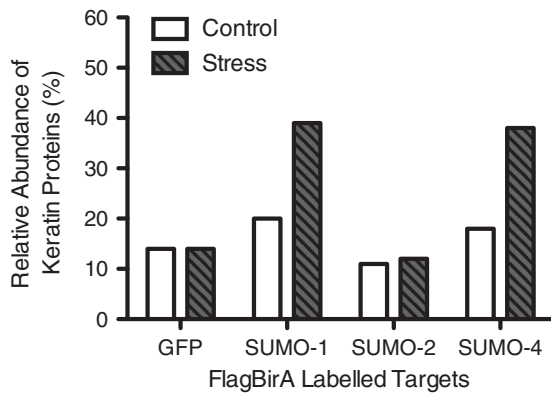

Figure 4. SUMO-1 and SUMO-4 association with keratins is enhanced under stress condition
Control and hyperosmotic stressed culture condition ratio of peptides assigned to keratin proteins over total peptides analysed per mass spectrometry analysis.

Discussion

Our data illustrate the importance of SUMOylation in normal human placental development and more importantly demonstrate that various cellular stressors have the ability to induce unique intra- and extracellular SUMO distributions in the placenta. Across gestation SUMO-2/3 is stably expressed in both the cytotrophoblast cells and the outer syncytiotrophoblast layer. However, SUMO-1 and SUMO-4 expression shifted from the cytotrophoblast to the syncytium as gestation
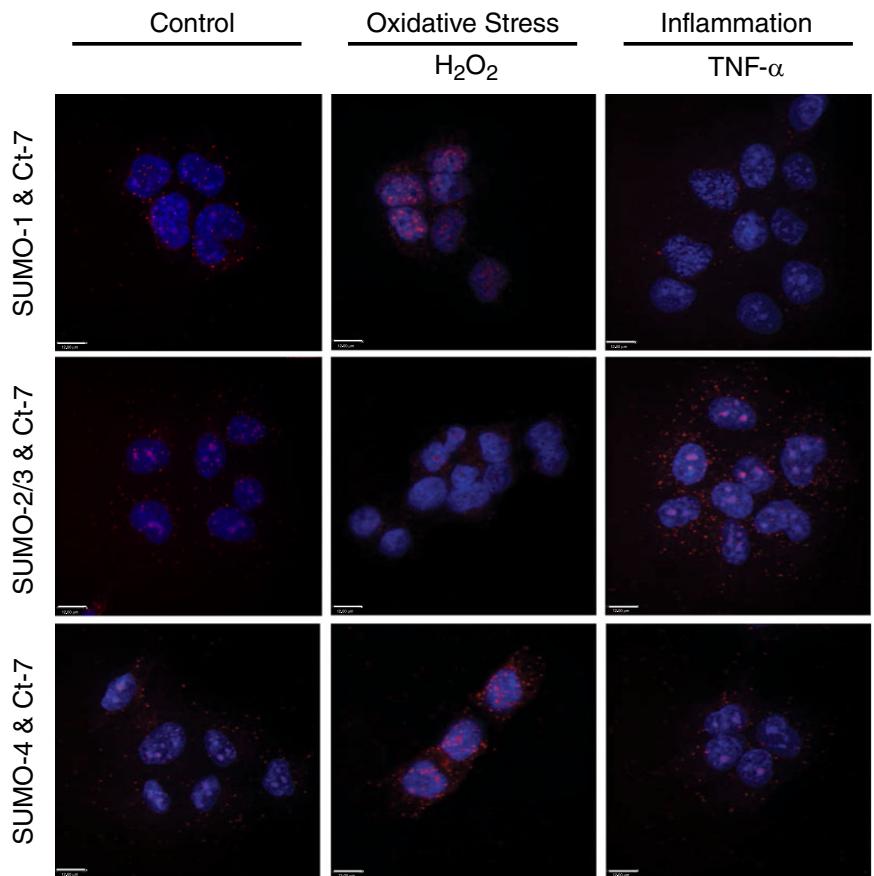
Figure 5. SUMO isoforms interact with cytokeratin-7 (Ct-7) in response to oxidative and inflammatory stress in in JAR cells
Cells were treated for 4 h with 0.1 mM H_2O_2 (oxidative stress) or 10 ng ml^{-1} TNF-α (inflammatory stress). H_2O_2 induced the interaction between SUMO-1 and Ct-7, as well as, SUMO-4 and Ct-7, whereas, TNF-α treatment stimulated the interaction of SUMO-2/3 and Ct-7. SUMO protein interactions with Ct-7 are labelled with red fluorescence and nuclei are counter-stained with DAPI (blue). Scale bar: 12 μm.

Table 1. Mass spectrometry demonstrates the relative abundance of SUMO conjugates with cytoskeleton filaments under control and stress (hyperosmotic) conditions in placental JAR cells

| | FlagBirA*-GFP | | | | FlagBirA*-SUMO-1 | | | | FlagBirA*-SUMO-2 | | | | FlagBirA*-SUMO-4 | | | |
| | Control | | Hyperosmotic | | Control | | Hyperosmotic | | Control | | Hyperosmotic | | Control | | Hyperosmotic | |
	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2
Total peptide counts	14734	14655	17262	17361	10092	9595	14696	14037	11574	11006	14245	15262	12498	12664	21368	21372
Keratin peptide counts	2061	2024	2482	2484	2050	1903	5867	5383	1289	1132	1630	2026	2501	2145	7484	8701
Tubulin peptide counts	305	326	471	432	213	220	449	403	220	241	320	351	271	267	650	577
Actin peptide counts	267	270	262	284	149	126	150	149	232	202	187	202	149	167	258	277
Proportion of keratin peptides (%)	14	14	14	14	20	20	40	38	11	10	11	13	20	17	35	41
Proportion of tubulin peptides (%)	2	2	3	2	2	2	3	3	2	2	2	2	2	2	3	3
Proportion of actin peptides (%)	2	2	2	2	1	1	1	1	2	2	1	2	1	1	1	1

Grey highlighting demonstrates increased relative abundance of SUMO–keratin conjugates in hyperosmotic cell culture conditions. GFP, green fluorescent protein; SUMO, small ubiquitin-like modifier protein.

progressed. When we examined models of oxidative stress (examined with hypoxia of 0.5% O_2 and H_2O_2 treatment), SUMO-1 and SUMO-4 cellular expression shifted from the cytotrophoblast to the syncytial cytoplasm, which was consistent with the observed localization of SUMO-4 in pre-eclamptic placentas. Analogous with localization seen in placentas complicated with chorioamnionitis, inflammatory stress resulted in an upregulation of SUMO-2/3 throughout the syncytium, especially within its cytoplasm Thus, these two discrete placental pathologies show very distinctive patterns of SUMO expression. Furthermore, this is the first report to identify keratins, specifically Ct-7, as a major placental-specific target of SUMOylation. Overall, our data support the hypothesis that individual SUMO isoforms have distinctive spatiotemporal roles in human placenta that may affect keratin function.

SENP knockout transgenic mouse models (Yamaguchi *et al.* 2005; Chiu *et al.* 2008) clearly demonstrate the importance of SUMOylation to normal placental development. The fact that all four SUMO isoforms are strongly expressed in the trophoblast layer of human placenta and show elevated levels in pre-eclampsia further implies a critical role for SUMOylation in placental function (Baczyk *et al.* 2013*a*, 2017). SUMO-1–4 immunostaining of placental tissue from

across gestation revealed a distinct distribution within the trophoblast layer. Specifically, SUMO-1 and SUMO-4 showed predominantly cytotrophoblast expression in first and second trimester with equal distribution to the syncytiotrophoblast in the third trimester. Conversely, SUMO-2/3 localization displayed a more even distribution throughout the trophoblast layer over gestation. In our *in vitro* experiments, hypoxic stress induced SUMO-1 and SUMO-4, but not SUMO-2/3 redistribution from the cytotrophoblast cell layer into the syncytium. This observation led us to postulate that the elevated SUMO-1 and SUMO-4 expression in the syncytium, observed with advancing gestation, may in part be due to increased cellular stress experienced by the ageing placenta. Other factors that are thought to be indicative of placental stress include alterations in angiogenic growth factors, such as soluble fms-like tyrosine kinase-1 (sFlt-1) and placenta growth factor (PlGF) (Redman & Staff, 2015). In healthy pregnancy as the placenta matures and reaches its maximum capacity for diffusional exchange, sFlt-1 levels increase while PlGF levels decrease during the third trimester (Redman & Staff, 2015). This time period correlates with observed changes in SUMO-1 and SUMO-4 redistribution from cytotrophoblast into the syncytium. Therefore, elevated SUMOylation in the syncytium, which is the only placental cell type in direct
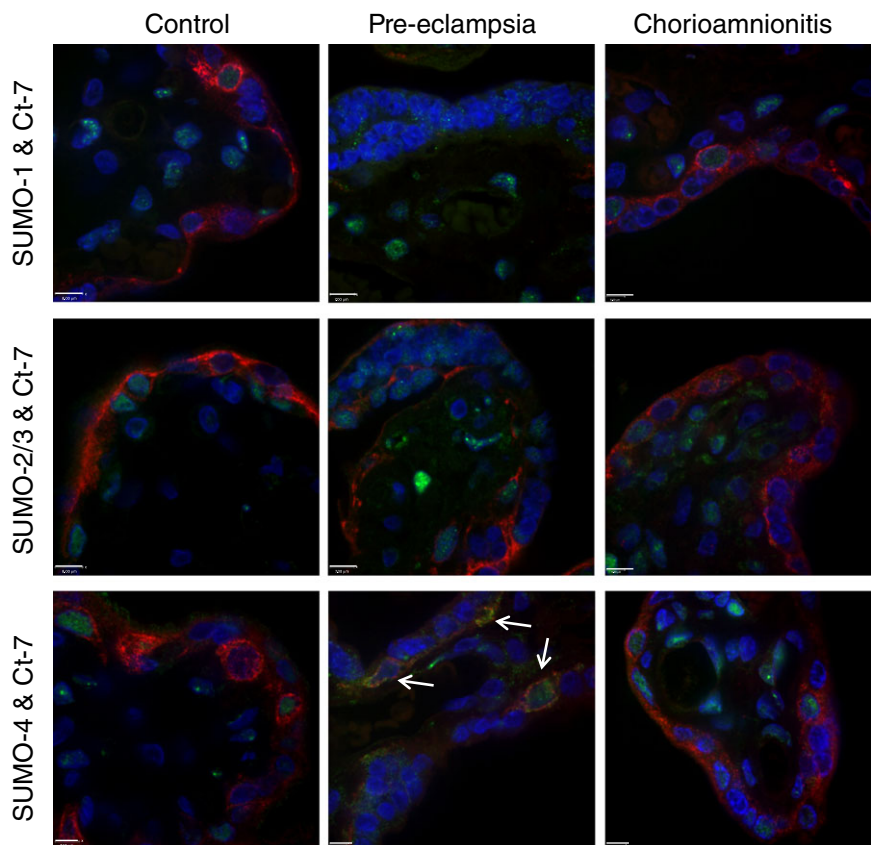
Figure 6. SUMO (green) and cytokeratin-7 (Ct-7; red) immunostaining in placentas from severe pre-eclampsia and severe chorioamnionitis

Keratin proteins are reduced in pre-eclamptic placentas compared to control and those with chorioamnionitis. SUMO-1 is predominantly cytoplasmic in pre-eclamptic placentas (29 weeks' gestation) and exhibited mild cytotrophoblastic perinuclear expression in chorioamnionitis (32 weeks' gestation). SUMO-2/3 cytoplasmic expression was increased in placentas with chorioamnionitis. SUMO-4 expression was increased in the cytoplasm of pre-eclamptic placentas and demonstrated co-localization with Ct7 (arrows). Representative images shown compared to age-matched control placentas (31 weeks); *n* = 4. Scale bar: 7 μm.

contact with maternal circulation, may be a consequence of external stress, but further studies would be required to delineate these changes.

Our data emphasize that individual SUMO isoforms have distinct functions, since they exhibit isoform-specific responses to placental stress. Intriguingly, JAR cells experience cytoplasmic accumulation of SUMO-1 and SUMO-4 with hypoxic and reactive oxygen species stimuli, whereas increased cytoplasmic SUMO-2/3 expression is observed with TNF-α treatment. Further studies could elucidate our observed trends, which would greatly enhance statistical power. Unique SUMOylation patterns are also observed in placentas from severe pre-eclampsia and chorioamnionitis, which are both pathologies attributed to distinct intrauterine stressors. Nuclear localization of SUMOylated proteins is known to affect their stability and transcriptional activity. SUMOylation has been shown to sequester proteins into nuclear bodies, thereby reducing their bioavailability and transcriptional activity (Chalkiadaki & Talianidis, 2005). Less is known about cytoplasmic SUMOylation of proteins but emerging evidence suggests that SUMOylation plays a very important role in the regulation of cytoskeletal proteins (Snider & Omary, 2014).

Our mass spectrometry analysis revealed that the most abundant targets of SUMOylation in the human placental JAR cells belong to the keratin family. Furthermore, stressing the cells doubled the keratin hits for SUMO-1 and SUMO-4, but not SUMO-2, which correlated with the predominantly cytoplasmic expression of SUMO-1 and SUMO-4 in response to hypoxic stress with no differences in SUMO-2/3. The proximity ligation assay demonstrated the interaction of SUMO-1 and SUMO-4, but not SUMO-2/3, with Ct-7 under oxidative stress and further supports our data implicating keratins as placental SUMOylation targets. It has been demonstrated that SUMO regulates the organization and solubility of intermediate filaments and cytoskeletal proteins (Snider & Omary, 2014). Intermediate filaments reorganize in response to stress and apoptosis and hyperSUMOylated intermediate filaments have reduced solubility and form high molecular mass complexes (Snider *et al.* 2011). In *C. elegans*, for instance, inhibition of SUMOylation reduced intermediate filament network turnover (Kaminsky *et al.* 2009), whereas hyperSUMOyation in human liver disease causes keratin insolubility (Snider *et al.* 2011). Our data reveal that in the human placenta, SUMOylation machinery might be linked to trophoblast cytoskeletal remodelling, and therefore the overall stability of this layer that is in direct contact with maternal blood.

Keratins are a vital component of epithelial cells, providing structural support; they also play a role in other cellular processes including protection from mechanical and non-mechanical stress (Gu & Coulombe, 2007) and tissue differentiation (Loschke *et al.* 2015). The role of keratins in placental development and particularly in placental pathology has not been completely elucidated. Riquelme *et al.* (2011) reported that human pre-eclamptic placentas demonstrate 50% down-regulation in Ct-7 expression and 20% down-regulation in β-actin expression as compared to healthy controls (Riquelme *et al.* 2011). Another group reported that keratins have reduced expression in the villous compartment of pre-eclamptic placentas as compared to healthy counterparts (Ahenkorah *et al.* 2009). Furthermore, these authors suggest that the down-regulation of keratins in pre-eclampsia results in 'cytoskeletally weaker' trophoblast, contributing to increased deportation of trophoblast debris into the maternal circulation. Pre-eclamptic placentas are characterized by having increased shedding of apoptotic and necrotic material from the trophoblast surface. We speculate that the hyper-SUMOylation, specifically by SUMO-1 and SUMO-4, could be involved in the mechanism regulating the stability of cytoskeleton filaments in human placenta. Since hyper-SUMOylation of keratins renders them insoluble (Snider & Omary, 2014) and hinders the nucleation step of the keratin cycle (Windoffer *et al.* 2011), it is also feasible to propose that the hyperSUMOylation observed in the pre-eclamptic placentas might contribute to alterations in the trophoblast turnover commonly seen in this condition.

Increased SUMOylation is presumed to elicit a protective cellular response by activating pro-survival pathways (Enserink, 2015); however, little is known about the effects of a prolonged hyperSUMOylation state. Snider & Omary (2014) provided overwhelming evidence in their chronic liver disease models that keratins 8, 18 and 19 are targets for SUMOylation, particularly under oxidative stress (Snider & Omary, 2014). In contrast to our data, they observed the strongest interaction of keratins with SUMO-2/3 and not SUMO-1. Furthermore prolonged stress and thus SUMOylation, did not correlate with reduced expression of keratins, only their elevated aggregation into high molecular mass complexes (Snider *et al.* 2011). Fully understanding the mechanism and consequences governing the SUMOylation of placental keratins is beyond the scope of this paper, but of great interest and requires further study.

In conclusion, numerous components of the SUMOylation machinery are uniquely expressed in the human placenta and show elevated levels in pre-eclampsia, suggesting that SUMOylation is a key component linked to underlying placental dysfunction. Our study critically links SUMOylation to keratins in the human placenta and provides evidence for the possible role of this inter-action underlying of dysfunctional trophoblast turnover. Whether hyperSUMOylation observed in pre-eclampsia is the cause or the effect remains to be determined. However, understanding the role of individual SUMO components

in placental development and function might aid us with tailored pharmacological interventions to modulate protein SUMOylation as a novel therapeutic approach for treating pre-eclampsia.

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Additional information

Competing interests

The authors declare that they have no conflict of interest.

Author contributions

D.B., M.A. and E.C. designed research, performed research, analysed data and wrote the manuscript. B.R. preformed interpretation of data analysis and critical revision of the manuscript. J.K. provided interpretation of data, critical revision of the manuscript and funding support for the study. All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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